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Evolutionary Cycles for Pericyclic Reactions – Or Why We Keep Mutating Mutases

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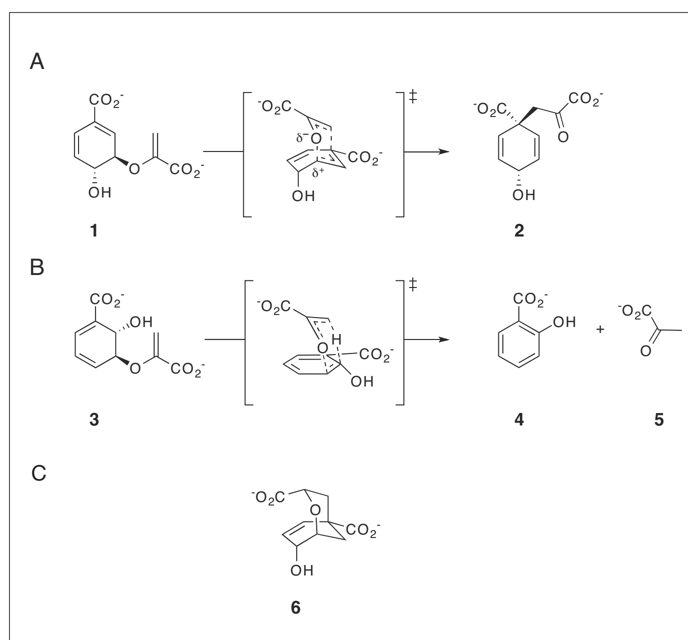
Abstract: Directed evolution strategies are being applied ever more frequently to develop novel and improved enzymes for many applications, including those contributing to 'white biotechnology'. In addition to engineering new biocatalysts, evolutionary strategies are equally suited to the elucidation of enzyme structure and function. Here, we illustrate with selected examples from our own work on chorismate mutases how such strategies can be employed to address a range of fundamental questions. Over the last decade, this model system, which was once considered to be a 'very simple' enzyme from the shikimate pathway, has afforded many – sometimes surprising – discoveries about biocatalysis. It has also taught us how to upgrade evolutionary approaches to overcome technical hurdles. Both the new insights and the methodological improvements should enhance our ability to tailor enzymes for novel uses.

Keywords: Chorismate mutase · Directed evolution · Enzyme structure and function · Isochorismate pyruvate lyase · Selection *in vivo*

Introduction

Life would not be possible without enzymes. These highly efficient catalysts facilitate even very difficult chemical transformations, thereby directing and running the complex metabolic network in each cell. Chorismate mutase (CM) is one of these enzymes; it catalyzes the Claisen rearrangement of chorismate (**1**) to prephenate (**2**) (Scheme 1A), a key step in the shikimate pathway leading to the aromatic amino acids phenylalanine and tyrosine.[6] Although the CM reaction looks rather simple and has been thoroughly studied for four decades, it is still hotly debated[7–10] what ultimately leads to the more than a million fold rate acceleration compared to the uncatalyzed process.[11]



Scheme 1. Reactions of chorismate mutase (CM) and isochorismate pyruvate lyase (IPL) and structure of a transition state analog. (A) CM catalyzes the Claisen rearrangement of chorismate (**1**) to prephenate (**2**) *via* a bicyclic, chair-like transition state.[1,2] (B) Pericyclic reaction mechanism for IPL to eliminate pyruvate (**5**) from isochorismate (**3**) to form salicylate (**4**).[3,4] (C) Bartlett's *endo*-oxabicyclic dicarboxylic acid **6**, the best inhibitor for most CMs.[5]

One of our research interests is the exploration of basic properties of enzymes by employing 'directed evolution'. This technology makes use of cycles of mutagenesis and amplification of selected variants, thereby mimicking Darwinian evolution, Nature's most powerful and successful strategy to create diversity. We chose CM as our study object because its reaction is unimolecular and does not involve cofactors, and very different CMs operating with similar efficiencies are available from Nature. The following examples with CMs demonstrate how evolutionary strategies can be used to examine mechanism and structure of enzymes and how readily catalytically active alternatives can be obtained.

How Do Enzymes Work? Clues from Directed Evolution

Both the uncatalyzed and the catalyzed CM reaction were shown to proceed *via* the same chair-like transition state shown in brackets in Scheme 1A.[1,2,12] The reaction is concerted, however with breaking of the C–O bond initiating before formation of the C–C bond.[13,14] To address mechanistic

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questions in CM catalysis, we implemented an evolutionary approach to explore the enzyme's active site as an alternative to conventional site-directed mutagenesis, which often leads to unpredictable outcome due to our still limited understanding of protein structure–function relationships.[15] Efficient directed evolution experiments rely on a powerful selection system, which we engineered for the CM reaction using specifically designed *Escherichia coli* mutant strains.[16] With this system, we probed the requirement and alternatives for prominent catalytic residues in the enzymatic Claisen rearrangement.[16,17] For instance, we demonstrated that arginine 90, which is juxtaposed to the ether oxygen of bound ligand (*e.g.* Bartlett's *endo*-oxabicyclic transition state analog **6**, Scheme 1C)[5] in the CM from *Bacillus subtilis* (BsCM), plays a crucial role in stabilizing the developing negative charge on the ether oxygen of the transition state.[16] The importance of electrostatic catalysis in BsCM was supported by X-ray crystallographic studies of mutants showing that a transition state-stabilizing positive charge can be delivered either by an arginine or lysine side chain.[18] Remarkably, these catalytic residues can successfully project from different locations in the protein chain into the active site, provided that sterically suitable compensatory mutations at other positions are selected from combinatorial sequence space.

Interestingly, one of the experimentally found alternative catalytic site configurations that features a lysine reaching into the active site was discovered subsequently in another natural CM, the CM domain of the bifunctional CM-prephenate dehydratase from *E. coli* (EcCM).[19] BsCM and EcCM represent fundamentally distinct and unrelated protein folds, with the former (the 'AroH class') consisting of a trimeric pseudo-α/β-barrel with the active sites at the subunit interfaces,[20] and the latter (the 'AroQ class') formed by two intertwined subunits that bury the ligands in α-helical bundle structures[19] (Fig. 1).

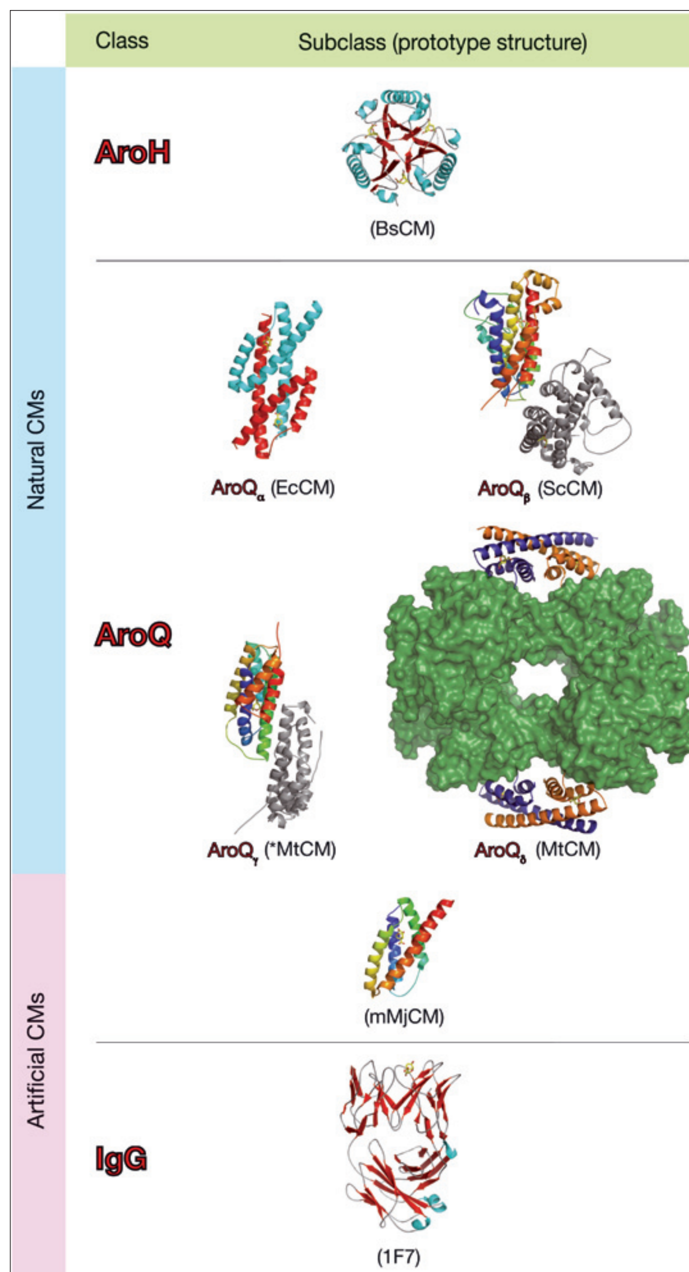

Fig. 1. Structural overview of natural and artificial folds known to catalyze the chorismate mutase reaction. AroH class CMs are naturally occurring pseudo-α/β-barrel enzymes with three active sites located at the interfaces of the subunits.[20] Four structurally distinct subclasses of the more abundant α-helical AroQ fold have been described so far, denoted $AroQ_{\alpha}$ through $AroQ_{\delta}$.[19,21–23] mMjCM, a monomeric but molten globular version of the thermostable CM from *Methanococcus jannaschii* was constructed artificially.[24,25] The catalytic antibody 1F7 (Fab fragment of the IgG fold shown)[26] is also able to catalyze the CM reaction. All structures are complexes with inhibitor **6** (in stick representation with yellow carbon atoms). The second CM subunit in a homodimer is shown in grey, if each protomer contains an independent active site; the tetrameric DAHP synthase core in the complex with $AroQ_{\delta}$ is shown in green surface representation. ScCM, allosterically controlled CM from *S. cerevisiae*.[22]

One Fold – Two Pericyclic Reactions

Together with the poorly active CM catalytic antibody 1F7, which was elicited against the transition state analog **6** as the hapten (Fig. 1),[26–28] the proteins from the AroH and AroQ class are exceptional in that they catalyze one of only very few known enzymatic pericyclic processes (for references, see ref. [3]). We were therefore thrilled to discover that the AroQ class protein PchB from *Pseudomonas aeruginosa* showed promiscuous activity; in addition to being a mediocre CM with a 1,000-fold lower catalytic efficiency than is typical, PchB is a highly active isochorismate pyruvate lyase (IPL). The IPL reaction involves the elimination of pyruvate (**5**) from isochorismate (**3**) to yield salicylate (**4**) (Scheme 1B), which is needed as a precursor in the biosynthesis of the siderophore pyochelin in *P. aeruginosa*.[29]

To distinguish between a [1,5]-sigmatropic rearrangement of **3** (Fig. 2A) and a general-base initiated proton abstraction followed by elimination of **5** (Fig. 2B) as the enzymatic mechanism, directed evo-

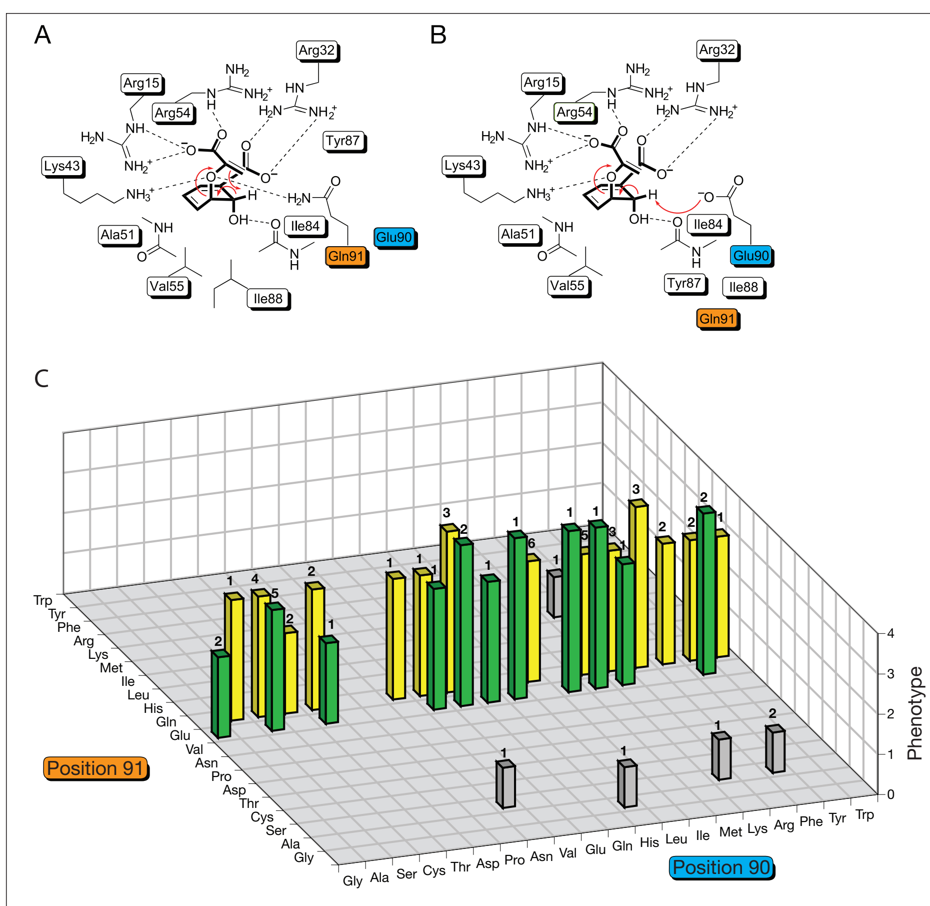

Fig. 2. Hypothetical isochorismate pyruvate lyase reaction mechanisms and their experimental investigation by randomizing active site residues. (A) and (B) Modeled active site of PchB with bound substrate **3**. The models were based on homology modeling with EcCM using alternative sequence alignments. (A) [1,5]-Sigmatropic rearrangement, facilitated by stabilization of developing charge on the ether oxygen of **3** by Lys43 and Gln91. (B) General-base mechanism proposed by Walsh and coworkers,[30] with proton abstraction by glutamate 90 and subsequent elimination. (C) Results of combinatorial mutational analysis of glutamate 90 and glutamine 91 reveal the model in (A) as consistent with the observed amino acid distributions in selected functional variants.[4]

lution experiments were carried out involving randomization of presumed key active site residues.[4] From the conservation patterns found in the selected active clones (Fig. 2C) we could derive that the reaction most probably proceeds as an asynchronous pericyclic process (Scheme 1B), facilitated by electrostatic stabilization of developing negative charge on the ether oxygen of **3** in the transition state, in analogy to the reaction of Scheme 1A (see previous section). Inhibition studies with **6** and kinetic characterization of individual mutants confirmed that both reactions occur in the same active site.[4] Furthermore, the fate of a deuterium atom at C(2) of **3**, kinetic isotope effects, and calculations are in clear support of the pericyclic nature of the IPL reaction,[3,31] thus adding another example to the still small set of enzyme-catalyzed pericyclic processes.

The residual CM activity of PchB suggests that its IPL function evolved from an ancestral AroQ CM.[29] Such a scenario became even more plausible, as we were able to 'retro-evolve' PchB into a 40-fold more efficient CM through only two cycles of directed evolution (D. Künzler, P. Kast, unpublished results). Our findings also hint at the AroQ fold being a potentially interesting starting point for the evolution of catalysts for other pericyclic reactions.

AroQ$_\gamma$ – A Novel Topologically Rearranged Fold

As shown above, directed evolution strategies are clearly useful to shed light on diverse mechanistic questions. Moreover, they can also elucidate structural features, as demonstrated here with the example of the secreted CM from *Mycobacterium tuberculosis*.

The α-helical AroQ class CMs split into several evolutionarily related subgroups, termed AroQ$_\alpha$ through AroQ$_\delta$,[21,23] which have quite distinct primary, tertiary and quaternary structures (Fig. 1). EcCM, described above, is a representative of the AroQ$_\alpha$ subclass. The AroQ$_\beta$ enzymes, found in eukaryotes such as the yeast *Saccharomyces cerevisiae*, are also homodimers made up of subunits composed of twelve α-helices each.[22,32] It was speculated that AroQ$_\beta$ CMs evolved from a duplicated primordial AroQ gene of the AroQ$_\alpha$ type, with one domain retaining catalytic function while the other one turned into an allosteric domain mediating regulation by the shikimate pathway end products tyrosine and tryptophan.[22]

When searching the genome of *M. tuberculosis* for genes encoding potential CMs, we did not find a candidate corresponding to any of the aforementioned structurally established enzymes. Instead, a protein (termed *MtCM) was present that exhibits some homology to AroQ$_\alpha$ CMs.[33] However, while *MtCM is about twice the size of an AroQ$_\alpha$ domain, it lacks the N-terminal half of the first α-helix (H1) and thus the crucial conserved catalytic site residue arginine 11 of EcCM that is believed to fix the distal carboxylate group of **1** in the appropriate position to facilitate the Claisen rearrangement.[19,34] Nevertheless, the dimeric *MtCM is a highly active CM.[33] Upon closer scrutiny, we found that a helix (H4) predicted in the middle of the *MtCM sequence aligned reasonably well with the missing part of H1 from EcCM.[33] By applying a directed evolution approach, we could show that the role of the missing arginine 11 of EcCM is assumed by arginine 134 in H4 of *MtCM (M. Gamper, S. Sasso, P. Kast, unpublished results). At the same time, other structurally important interactions were probed in this evolutionary experiment, and the results led to the conclusion that *MtCM is a topologically rearranged variant of the prototypical AroQ$_\alpha$ fold. We thus assigned *MtCM to a new subclass termed AroQ$_\gamma$. Our structural assignments are in agreement with the crystal structure of *MtCM (Fig. 1), which we subsequently solved to 1.6 Å resolution with and without transition state analog **6**, and which represented the first structure of an AroQ$_\gamma$ CM.[21]

The most striking feature of AroQ$_\gamma$ CMs is, however, that they are produced with an N-terminal signal sequence, resulting in the translocation of the enzyme out of the cell.[33] This is particularly puzzling as the entire shikimate pathway is localized intracellularly. Remarkably, there seems to be a correlation between the pathogenicity of an organism and the presence of an AroQ$_\gamma$ subclass CM in that organism, whereas the infected host does not necessarily need to possess the shikimate pathway (for references, see ref. [33]). For instance, *M. tuberculosis*, which persists as an intracellular pathogen in the vacuole of human lung macrophages, probably secretes *MtCM into its host, even though **1** and **2** are not

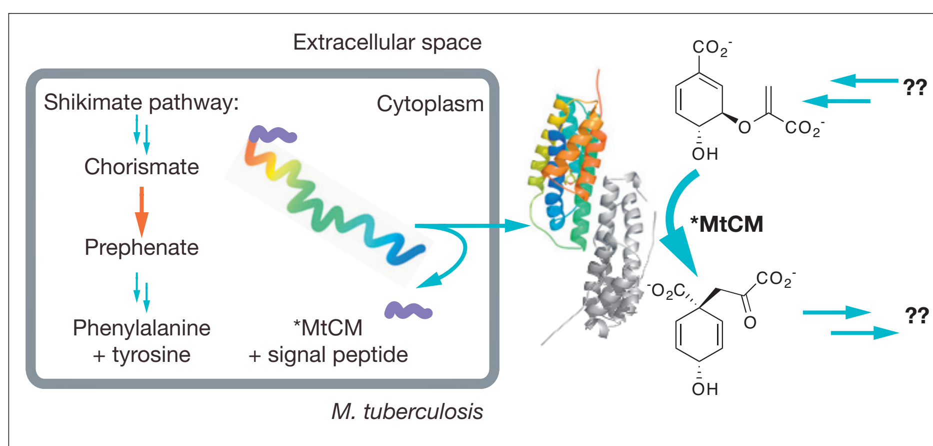

Fig. 3. The AroQ$_\gamma$ fold protein *MtCM is secreted from the Gram-positive bacterium *M. tuberculosis*.[35] The bacterial shikimate pathway is entirely located in the cytoplasm; nevertheless, *M. tuberculosis* produces an extracellular CM.[33] Its biological function, the source of its substrate **1** and the fate of the reaction product **2** are still under investigation.

metabolites in mammals (Fig. 3). It will be interesting to unravel the biological significance of these secreted AroQ$_\gamma$ CMs.

Chorismate Mutases Can Be Surprisingly Complex

Since the exported *MtCM is obviously not the housekeeping CM in *M. tuberculosis*, we again searched the genome sequence for CM candidates. A second gene showing similarity to the *aroQ* gene family was discovered that codes for a short protein of 90 amino acid residues (termed MtCM). However, MtCM is truncated on the C-terminal side compared to the prototypical AroQ$_\alpha$ CMs, and thus lacks a counterpart of the important catalytic residue glutamine 88 of EcCM. Furthermore, an arginine takes the position of the consensus lysine 39, which in EcCM together with glutamine 88 can stabilize developing charge on the ether oxygen of **1** in the transition state.[19,34] When we determined the enzymatic activity of MtCM it turned out to be two orders of magnitude below that of a typical CM,[23] in contrast to the very active exported *MtCM.[33]

Such reduced activity is highly unusual for a naturally evolved metabolic enzyme. We therefore looked for potential activating factors and – particularly after our experience with the IPL activity of PchB – for an associated major activity. However, Nature's solution to this puzzle turned out to be the involvement of MtCM in an activating non-covalent complex with another shikimate pathway enzyme, the D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase. Complex formation increased CM activity to normal levels and endowed MtCM with regulatory properties by making it subject to metabolically meaningful feedback inhibition by phenylalanine and tyrosine.[23] We determined the crystal structure of MtCM alone and of MtCM (with and without **6**) in the octameric complex with DAHP synthase at high resolution (Fig. 1) and suggest a model for the mechanism of CM activation.[23] We are currently exploring the idea that disrupting this complex, which apparently only occurs in the bacterial order *Actinomycetales* (including mycobacteria), could represent a promising and specific drug target.

MtCM also represents an interesting system to study how easily the activity of a naturally sluggish enzyme can be enhanced. To this end, MtCM was subjected to directed evolution in the absence of its complex partner (Fig. 4A). After only two evolutionary cycles, a variant with two mutations was found that showed an increase in the catalytic efficiency of an order of magnitude, demonstrating the potential of the evolutionary strategy (S. Sasso, P. Kast, unpublished results). Each mutation enhanced k_{cat}/K_m by about a factor of 3–4. The observation that one of them (V62I) was actually in the active site, and the other one (A36T) in a more remote location at the dimer interface, is in agreement with our long-term experience that beneficial mutations can occur anywhere in the enzyme, and often at an unexpected position (Fig. 4B). Additional evolutionary experiments resulted in MtCM variants with catalytic efficiencies within a factor of 5 of typical wild-type CMs (K. Roderer, P. Kast, unpublished results).

Evolution of Highly Active Artificial Chorismate Mutases

As observed with the best MtCM variants, an obvious end point of any directed evolution experiment is reached, if the catalyst is so efficient that no further selection pressure can be applied. Such a situation can be circumvented by reengineering the selection system, as exemplified below.

By inserting short peptide loop sequences into the first helix (H1) of the prototypic homodimeric AroQ$_\alpha$ fold, we have evolved artificial CMs that have quaternary structures ranging from a highly active, but molten globular monomer (Fig. 1) to a hexameric species.[24,36] When evolving the hexameric variant of EcCM further, we faced the problem that after two evolutionary cycles our selection system could not differentiate anymore between wild-type EcCM and the best variant (tEcCM), even though the variant's k_{cat} was still 14 times lower. In principle, several strategies can be applied at this point to increase selection pressure *in vivo* for any target reaction that has a metabolic impact. These strategies include the manipulation of the intracellular concentration of relevant metabolites or of active catalysts. For instance, if the target enzyme's substrate is an endogenous metabolite, its intracellular concentration could be reduced by channeling it down a competing metabolic branch.[37]

The intracellular catalyst concentration can be influenced in a number of ways, such as variation of the gene dose through altering plasmid copy number,

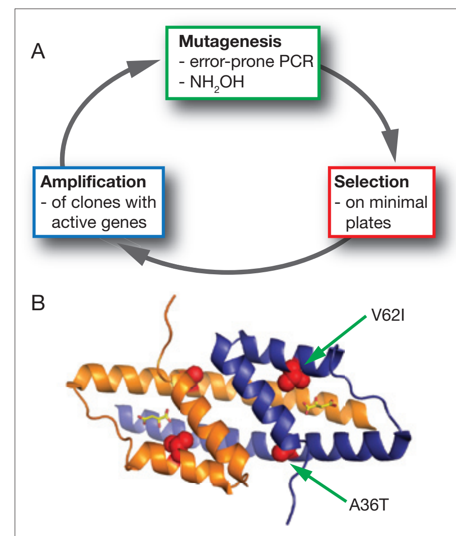

Fig. 4. Directed evolution consisting of cycles of random mutagenesis, selection, and amplification is employed to evolve better catalysts. (A) Evolutionary scheme used to evolve better variants of the naturally occurring MtCM from *M. tuberculosis*. PCR, polymerase chain reaction. (B) Mapping of two catalytically beneficial mutations in the crystal structure of the MtCM dimer. Mutated residues are shown as red CPK models; a malate ion bound to the active site is shown in stick representation. Beneficial mutations can be in the active site (valine 62 to isoleucine, V62I) or elsewhere in the protein, for instance, at the dimer interface (alanine 36 to threonine, A36T), where they could influence protein packing (S. Sasso, P. Kast, unpublished results).

regulation of gene transcription using inducible promoters, altering translation efficiency by exchanging the ribosomal binding site, and proteolytic turnover of active catalysts. To increase the selection pressure for evolution of still better variants of tEcCM, we implemented a combination of a tighter, better regulatable promoter system (P_{tetA} promoter) and a further lowering of intracellular tEcCM concentration through the appended eleven amino acid SsrA tag for rapid proteolytic degradation (Fig. 5). With this modified selection system it was possible to further improve the catalytic efficiency of tEcCM in only one evolutionary round to match wild-type EcCM.[38]

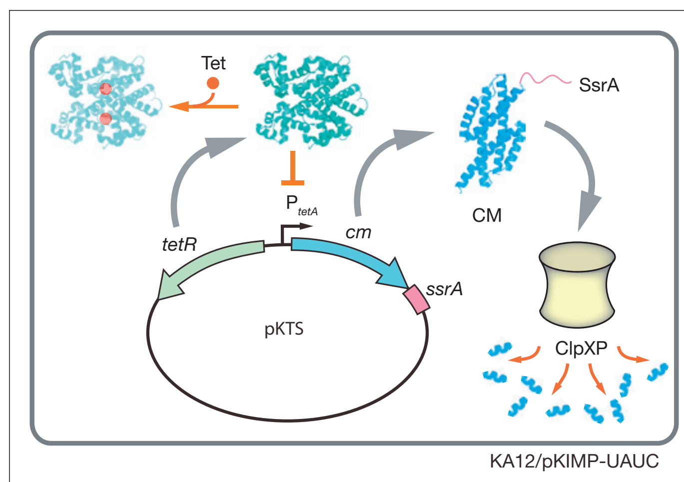

Fig. 5. A tetracycline-inducible promoter system in combination with a degradation tag for controlling catalyst concentration in directed evolution experiments.[38] The tetracycline (Tet)-inducible promoter P_{tetA} provides graded and homogeneous gene expression in our chorismate mutase selection system KA12/pKIMP-UAUC. Additional selection stringency is obtained by attaching an SsrA degradation tag to the C-terminus of the protein of interest, directing correspondingly equipped proteins to the ClpXP protease. The resulting lowered intracellular catalyst concentration is advantageous for selection of highly active enzymes. Plasmid pKTS carries the genes *tetR* (repressor of the *tetA* promoter in absence of Tet) and, in this case, a CM gene (*cm*) fused to the *ssrA* sequence.[38]

Conclusions

Most chemical reactions in biology rely on enzymes. Therefore, great efforts are being invested to understand them and – from an engineering point of view – to make new ones that are as good as Nature's, or even better. The examples from our laboratory described here illustrate that we can still learn many different lessons by dissecting a seemingly innocuous enzyme. It became apparent, for instance, that (i) there exist many alternative solutions for a given catalytic task, (ii) enzyme-catalyzed pericyclic reactions may occur in preferred folds and may be more abundant than previously thought, (iii) structural information can be deduced from selection for function, and (iv) a combination of different strategies to control intracellular catalyst concentration can make evolutionary systems more rigorous. By continuing research in our laboratory on the CM system, we expect to further advance the technology of directed evolution, for instance by implementing robotics, and to explore the utility of the AroQ scaffold for different applications.

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